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Comparison of milk and serum enzyme–linked immunosorbent assays for diagnosis of *Mycobacterium avium* subspecies *paratuberculosis* infection in dairy cattle

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Abstract. Milk and serum samples from 35 dairy herds in 17 states were evaluated for cow- and herd-level *Mycobacterium avium* subspecies *paratuberculosis* (MAP) antibody test agreement. Evaluation of 6,349 samples suggested moderate agreement between milk and serum enzyme–linked immunosorbent assay (ELISA) results, with a kappa value of 0.50. Cow-level sensitivity (Se) for 18 dairy operations with 1,921 animals was evaluated relative to fecal culture results. At the cow level, the milk ELISA relative Se was not significantly different from that of the serum ELISA (21.2 and 23.5%, respectively). Logistic regression models revealed a positive association between lactation number and milk ELISA status. Non-Holstein cows were more likely to test milk ELISA positive than Holstein cows. Cows in the first 2 weeks of lactation and after week 45 of lactation were more likely to test milk ELISA positive than cows between 3 and 12 weeks of lactation. Milk production > 80% of herd average was negatively associated with testing milk ELISA positive. Animals in the West and Midwest regions were less likely than animals in the Southeast region to test ELISA positive by either test. Estimates for herd-level sensitivity for the milk and serum ELISA, relative to fecal culture results, ranged from 56 to 83%. At the cow and herd levels, milk ELISA performed equivalent to serum ELISA using fecal culture as a reference for MAP infection and has the advantage of decreased labor costs on farms that use Dairy Herd Improvement Association testing.

Key words: Enzyme-linked immunosorbent assay; milk; *Mycobacterium avium* subspecies *paratuberculosis*; paratuberculosis.

Introduction

Johne's disease is a chronic disease of dairy cattle that causes diarrhea, weight loss, and decreased production in affected animals. *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is the causative agent of Johne's disease.[4] As reported in the 1996 dairy study by the National Animal Health Monitoring System (NAHMS) of the US Department of Agriculture (USDA),[19] the apparent prevalence for Johne's disease at the animal level was 3.4% on the basis of results of a serum enzyme-linked immunosorbent assay (ELISA). The same study estimated the US dairy herd-level prevalence to be between 17 and 41%. MAP infection has been estimated to cost the US dairy industry more than $200 million annually.[22]

The NAHMS Dairy 2002 study reported that serum ELISA and fecal culturing were used to evaluate MAP infection at approximately 24 and 8% of operations, respectively.[29] These traditional Johne's disease testing methods are characterized by low sensitivity and high specificity.[5,6,25] Compared with fecal culturing, the serum ELISA is quick and relatively easy to perform. However, with both testing methods individual samples are collected and used almost exclusively for MAP testing.

The milk ELISA is similar to the serum ELISA in terms of testing time and cost. Since milk samples from individual cows are routinely collected on dairy farms enrolled in Dairy Herd Improvement Association (DHIA) testing, the milk ELISA may prove to be a less labor-intensive method for testing dairy cattle for MAP infection, compared with the serum ELISA. In addition, the routine collection and testing of milk samples would allow producers to more consistently screen their herds for infection without the additional scheduling of serum or feces collection. Findings from studies comparing the efficacy of individual animal milk ELISA and serum ELISA have varied. One study, using a lipoarabinomannan (LAM) ELISA, indicated that the milk LAM ELISA was slightly more accurate than the serum LAM ELISA.[24] A study from Germany[32] indicated signif-

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icant correlation between serum and milk ELISA results. Using the serum ELISA as a reference, the reported relative sensitivity and specificity of the milk ELISA was 60.9 and 94.6%, respectively. This milk ELISA is now licensed in Germany.

In a Missouri study,[11] however, it was reported that the milk ELISA used for detecting MAP exposure lacked correlation with the serum ELISA. Regression models indicated no differences in age, lactation number, peak milk production, 305-day milk average production, and linear somatic cell count, relative to milk ELISA status. Longitudinal evaluation of the milk ELISA in Denmark revealed milk antibody levels to be generally higher at the beginning and end of lactation.[21] Although there was variation on the basis of lactation stage, the authors, on evaluating individual cow results, reported stable antibody levels throughout lactation, with some cows having higher values than others. A cross-sectional study in Denmark using paired samples also indicated that the probability of cows testing milk ELISA positive was higher at the beginning and at the end of lactation.[20]

A commercial milk ELISA is available in the USA; however, to the authors' knowledge, validation and licensing of the test have not been completed. Two recent studies[6,13] indicated the sensitivity of this commercially available milk ELISA to be comparable to the sensitivity of the serum ELISA, relative to that of fecal culturing. Agreement between milk and serum ELISA results was reported as moderate to high in both studies. The milk ELISA has the potential to be used in Johne's disease control programs to identify cow MAP shedding and at high risk for transmitting MAP, estimating herd prevalence, or herd-level screening, or all 3 of the aforementioned functions. Further research on cow-level factors could increase the effectiveness of implementation by determining the stage of lactation when cattle are mostly likely to test positive.

The first objective of the study reported here was to compare the performance of milk ELISA, serum ELISA, and fecal culturing methods for diagnosing MAP infection in individual dairy cows and at the herd level for operations across the USA. The second objective was to evaluate cow-level factors associated with testing ELISA positive in a subset of dairy cattle operations participating in the NAHMS Dairy 2002 study.

Materials and methods

State and operation selection

A goal of the NAHMS Dairy 2002 study was to include states that accounted for at least 70% of the nation's dairy cows and 70% of operations with milk cows. National Agricultural Statistics Service data were used to determine the major US dairy states on the basis of dairy cattle populations. Final selection for phase I of the study included 21 states from 4 regions, representing 85.5% of milk cows in the USA and 82.8% of operations with milk cows.[28] A stratified random sample of operations in the 21 participating states was selected on the basis of the number of milk cows on the operations and the operator's willingness to participate in phase II of the study.

A subset of herds participating in phase II was invited to participate in within-herd MAP prevalence testing—using serum ELISA and fecal culturing—on the basis of operation's risk of having and transmitting the organism. Of this subset, those enrolled in Dairy Herd Improvement Association (DHIA) testing were asked to participate in additional MAP testing and allow access to individual milk samples and production records for analysis. Thirty-five operations from 17 states participated in milk ELISA testing. Operations from the following regions (and states) were included in the study: West (California, Colorado, New Mexico, Texas, Washington); Midwest (Indiana, Michigan, Minnesota, Missouri, Ohio, Wisconsin); Northeast (New York, Pennsylvania, Vermont); and Southeast (Florida, Tennessee, Virginia). Herds in Idaho, Iowa, Illinois, and Kentucky did not participate in the milk ELISA study.

Animal selection

A cross-sectional study performed in the 21 participating states included the initial survey and the sampling of individual cows for Johne's disease testing. Cows in the second or greater lactation were targeted for serum ELISA and fecal culturing for MAP. The testing scheme was designed to detect within-herd prevalence of MAP infection of $\geq 2\%$ with 95% confidence. Published statistical sample size recommendations, which are based on lactating herd size, were used.[30] The milk ELISA was performed on all lactating cows in the herd at the time of DHIA testing.

Sample collection and testing procedures

Serum and fecal samples were collected by USDA Animal and Plant Health Inspection Service-Veterinary Services (APHIS-VS) personnel on participating farms from March 25 to September 25, 2002. Cows in second or greater lactation were targeted for serum and feces testing. At the time of sample collection, cows were scored for body condition (thin, normal, or fat) and fecal consistency was evaluated (normal, loose, or watery). Samples were shipped to the National Veterinary Services Laboratories in Ames, Iowa. Serum and fecal samples were stored at -20 and -70°C, respectively, until tested.

All serum samples were tested for antibodies against MAP using a commercially available ELISA[a] according to manufacturer's recommendations, with the exception that samples were only tested in a single well. Test results were categorized as negative or positive on the basis of the kit manufacturers' recommendations. ELISA scores were used to further classify positive tests as positive or strong positive on the basis of guidelines from the Wisconsin

Table 1. Classification and interpretation of results of an ELISA* for antibodies against *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in dairy cows.†

Result	ELISA score	Explanation and recommendation
Negative	0.00–0.49	Antibodies against MAP were not detected. Cows are either not infected or not producing antibodies.
Inconclusive	0.50–0.99	Cows are more likely to be infected than cows with negative results. Retesting is recommended.
Positive	1.00–3.49	Cows are approximately 30 to 75 times as likely to be infected with MAP as are cows with negative results.
Strong positive	≥3.50	Cows are approximately 175 to 200 times as likely to be infected with MAP as are cows with negative results. Cows have a higher probability of developing clinical paratuberculosis in the next 12 months than do cows with lower scores.

* Paracheck, Biocor Animal Health, Omaha, NE.

† Adapted from Wisconsin Veterinary Diagnostic Laboratory, University of Wisconsin–Madison Interpretation Chart.

Veterinary Diagnostic Laboratory (Table 1). ELISA scores were calculated for each sample by subtracting the mean optical density (OD) of the negative control from the OD of the test sample, and then multiplying this difference by 10.

Fecal samples were cultured by 3 methods (Herrolds egg yolk [HEY] agar, BACTEC 460,[b] and Trek (ESP) Culture System II[c]) run in parallel as previously described.[15,29] Briefly, 2 g of specimen was resuspended in 35 ml of distilled water and shaken to disperse visible clumps. After 30 min, a 5-ml aliquot was removed and transferred to a solution containing 0.9% (w/v) hexadecylpyridium chloride in half-strength brain heart infusion (BHI) broth. After overnight incubation, the specimens were centrifuged at 3,000 × *g* for 20 minutes, the supernatant was discarded, and the pellet was resuspended in 1 ml of half-strength BHI containing vancomycin (100 μg/ml),[d] amphotericin B (50 μg/ml),[d] and nalidixic acid (100 μg/ml).[d] Prior to inoculation, each BACTEC 12B bottle, containing Middlebrook 7H12 broth, was supplemented with 0.2 ml of a solution containing polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin[e]; 0.1 ml of mycobactin J solution (50 μg/ml); 0.7 ml of sterile deionized water; and 1.0 ml of a 50% egg yolk solution (1 : 1 ratio of sterile egg yolks and sterile phosphate buffered saline [PBS]). Similarly, each bottle of a modified Middlebrook 7H9 broth[f] and compressed sponges, was supplemented with 1.0 ml of a Middlebrook OADC enrichment medium containing bovine serum albumin, dextrose, oleic acid, catalase, and sodium chloride[g]; 0.5 ml of a proprietary mixture of vancomycin, amphotericin B, and nalidixic acid[h]; and 1 ml of a proprietary suspension of egg yolk enrichment manufactured from fresh antibiotic-free eggs.[i]

After an additional overnight incubation of the inoculum, appropriately supplemented ESP II and BACTEC 12B liquid culture bottles were aseptically inoculated with 0.75 ml and 0.1 ml of the treated specimen, respectively. In addition, approximately 50 μl of the same decontaminated inoculum was swabbed onto each of 2 tubes of HEY agar containing mycobactin J, and 1 tube of HEY without mycobactin. All suspect colonies on solid media and samples that were signaled as positive by the BACTEC 460 or Trek culture methods were confirmed as MAP by use of polymerase chain reaction (PCR) methods.

For this, DNA from suspect positive cultures was prepared using a kit,[j] with the following modifications. For bacterial colonies on solid media, a single colony was resuspended in 1.0 ml of sterile PBS and vortexed briefly. Similarly, for the BACTEC and ESP II liquid cultures, 0.5 ml of broth was mixed with 1.0 ml of sterile PBS and vortexed. Samples were then centrifuged at 6,000 × *g* for 5 min, the supernatant was removed, and the sample was then treated as per the manufacturer's protocols.

To perform real-time PCR analysis, the IS*900* regions of the MAP genome were targeted using the primers 3-F (5′-ccg cta att gag aga tgc gat tgg-3′) and 3-R (5′-aat caa ctc cag cag cgc ggc ctc g-3′), and the 5′-fluorescein phosphoramidites (FAM)-labeled fluorescent probe IS900P (5′-tcc acg ccc gcc cag aca gg-3′) as previously described.[15] Each specimen was tested in duplicate wells of a 96-well plate, using a total of 5 μl of extracted DNA in a 25-μl reaction, prepared with a universal master mix as supplied by the manufacturer.[k] Thermocycling profiles consisted of an initial 10-min denaturation step at 95°C, followed by 40 cycles of a 25-sec denaturation step at 94°C, plus a 1-min annealing/extension step at 66°C.

A sample was reported as positive if it was positive by use of 1 or more test methods. The amount of MAP in an individual sample was quantified into a shedding level on the basis of previously reported guidelines.[29] Briefly, all positive fecal samples were classified into semiquantitative categories of high, moderate, low, and very low on the basis of the parameters described in Table 2. If discordant results were observed by use of 2 methods for the same isolate, the most conservative shedding category was chosen. Similarly if discordant results were observed by use of 3 methods for the same isolate, the shedding category was assigned on the basis of agreement by 2 of the 3 methods.

DHIA personnel collected milk samples from all lactating cows at each operation during routine milk sampling between June 1, 2002 and January 9, 2003. To preserve them during shipment and testing, milk samples were treated with bronopol according to manufacturer instructions.[l] Milk samples were collected from 10 days before to 267 days after serum and fecal collections. Approximately 50% of milk samples were collected within 2 months of collection of serum/fecal samples, and 80% of milk samples were collected within 4 months.

Table 2. Semiquantitation parameters for fecal culture results by culture method.

Culture system	Semiquantitation categories*			
	High	Moderate	Low	Very Low
BACTEC 460	GI > 300 at ≤3 wk	GI > 300 at 4–5 wk	GI > 100 at 6 wk	GI ≤ 100 at 6 wk
ESP II	<21 days to signal	22–28 days to signal	29–35 days to signal	36–42 days to signal
HEY	>50 cfu/tube	5.1–50 cfu/tube	0.5–5 cfu/tube	NA

GI = growth index; HEY = Herrolds egg yolk medium; cfu = colony-forming units; NA = not applicable.
* Semiquantitative estimation of bacterial load present in positive fecal specimens on the basis of parameters established for each culture method.

Milk samples were shipped for testing from multiple DHIA testing laboratories to a commercial diagnostic laboratory.[m] Most of the samples were stored frozen (−20°C), and testing was completed within 1 week. Individual milk samples were first screened (100 µl of mixed whole milk/well) by use of an indirect ELISA on whole milk samples, and samples reacting in the screening assay (0.07 absorbance units above that for negative controls) were retested in duplicate (100 µl of preabsorbed mixed whole milk/well) in an absorbed confirmation assay[6,13] for routine use on milk samples submitted to the diagnostic laboratory.[m] The screening assay has a sensitivity of 99% and specificity of 62%, compared with the confirmation assay (unpublished data[m]), and is designed to improve throughput and reduce cost of analysis of DHIA milk samples, compared with that of the confirmation ELISA alone.

The antigens used in the milk ELISA were derived from a proprietary strain of *Mycobacterium* grown in supplemented Watson-Reid medium[18] for 8 weeks or equivalent density. Bacteria were subsequently harvested at stationary phase by centrifugation, washed 3 times in PBS and heat treated,[23] and the supernatant was then clarified through a 0.22-µm filter. For the screening assay, the protein concentration of the supernatant was determined, and the crude antigen preparation was diluted in carbonate buffer (pH 9.6) and adsorbed onto assay plates[n] at a concentration of 300 ng/well. For the confirmation assay, the antigen was further purified by trichloroacetic acid precipitation (4%), resuspended in PBS, and diluted in carbonate buffer at a concentration of 300 ng/well.[23] After aspiration, the wells were postcoated with a commercial blocking and stabilizing reagent[d] and dried.

Unlike the screening assay where whole milk was used directly on the assay plates, mixed whole milk samples for the confirmation assay are first absorbed (1 : 1 dilution) for 30 minutes in a phosphate buffer containing antigens derived from a proprietary strain of *M. phlei*. Briefly, *M. phlei* were grown in supplemented Dorset and Henley Medium[17] for 2 weeks and the bacteria were harvested, washed, and treated similarly to those of the aforementioned crude antigen. Working concentrations were determined by checkerboard titrations[8] against banked samples known to contain interfering, reactive substances. Stock concentrations were back-calculated to deliver appropriate absorbing antigen in a 1 : 1 dilution of milk samples to eliminate all cross-reactive substances represented in the sample bank.

The conjugate for the assays was a horseradish peroxidase-derivatized monoclonal antibody against IgG1 purchased from a commercial source.[d] Working conjugate concentrations in PBS-Tween were determined for each antigen preparation in checkerboard titrations against standard positive and negative reference samples. Optimal dilutions maximized the ratio of positive to negative reference sample OD values with negligible reactivity of the negative reference (OD < 0.10) after a 10-min incubation in standard tetramethylbenzidine (TMB) substrate solution (0.2 mg of citrate buffer/ml, pH 4.0). Stock concentrations were determined by back-calculation and were stabilized with 1% bovine serum albumin.

The screening and confirmation assays were run similarly at ambient room temperature. Briefly, samples (mixed whole milk with or without absorption) were incubated in antigen-coated plates for 30 minutes. The wash steps between all incubations consisted of 5 wash cycles using 300 µl of PBS-Tween/well. The conjugate solution (100 µl) was added and allowed to incubate for 30 min; then it was washed, followed by the addition of 100 µl of TMB substrate. After 10 minutes, the reaction was stopped by the addition of 0.5 N sulfuric acid, and the OD values for samples and standards were determined at 450 nm.

The milk ELISA dataset used for the present analysis consisted of sample OD, negative and positive control ODs, and scores ([sample OD – negative control OD] × 10) from the screening assay of samples that did not react in the screening assay, and the same information from the confirmation assay of all samples that reacted in the screening assay. Milk ELISA score > 0.7 was considered a positive result. Although samples were only categorized as positive or negative, samples with ELISA score ≥ 3.5 units were considered as strong positive for portions of this analysis.

Production data

DHIA records, which contain numerous production-related variables, were obtained directly from the Dairy Record Processing Centers (DRPC) on a monthly or bimonthly basis. Data were collected for the entire lactation in which animals were tested. Commercially available dairy management software packages[o–q] were used to extract data from complete herd records. Parameters collected for analysis included breed, lactation number, days since calving at sample collection, and relative herd-level milk production (cow rating). Cow rating was calculated for an

individual cow by converting to energy-corrected milk (ECM) and dividing the cow's ECM value by the ECM lactation average for the herd. If a herd was composed of multiple breeds, adjustments were applied at the DRPC when calculating ratings. Days since calving was categorized into 5 lactation periods: 1–2 weeks, 3–12 weeks, 13–28 weeks, 29–44 weeks, and 45 weeks or longer.

Statistical analysis

Cow level. Pearson correlation was used to compare the ELISA scores of both tests. Overall test agreement was evaluated using kappa statistics.[9] Proportion agreement was also evaluated for positive, negative, and all samples. Stuart's test statistic was calculated for 3 outcome comparisons (negative, positive, and strong positive). Calculations of the relative sensitivity of the milk ELISA to the serum ELISA and of both ELISA methods, compared with fecal culture results, were performed. Statistical analyses were conducted using SAS.[r]

Two logistic regression models were developed in SUDAAN statistical software[s] to determine animal-level characteristics that increased the odds of testing milk or serum ELISA positive. The analysis accounted for the potential clustering effect of test-positive animals within herds. Breed, lactation number, lactation period (categorized by days in milk at time of sample collection), body condition score, fecal consistency, cow rating (relative herd-level milk production), and region (West, Midwest, Northeast, Southeast) as well as all 1-way interactions were evaluated as explanatory variables. A stepwise backward elimination process was used to create the models, and variables with Wald F statistics with $P < 0.05$ were considered significant. Main effects were not removed from the model if included in an interaction, regardless of P-value. Model fit was evaluated on the basis of the Hosmer-Lemeshow chi-square test P-value.[14]

Herd level. Comparing test-positive animals with the total tested within a herd for each testing modality was used to estimate within-herd prevalence. Apparent within-herd prevalence estimates for both ELISA methods were calculated and compared using Pearson correlation methods.

Agreement between the milk and serum ELISA herd-level results was calculated using kappa statistics and proportion agreement. To consider a herd infected, 2 test prevalence cutoff levels were evaluated by use of ELISA. Initially, if any animal tested ELISA (either milk or serum) positive, regardless of herd size, the herd was classified as infected. A 2% within-herd prevalence also was chosen as a cutoff level of the ELISA being evaluated since the calculated specificity of both ELISAs relative to fecal culturing was 98–99%, and this is a commonly reported specificity for ELISA methods.[5,6,7,25] Herd-level sensitivity (HSe) was calculated for the milk, compared with the serum ELISA results, and with fecal culture results. The relative HSe of serum ELISA results also was calculated using fecal culture results as the reference. Only herds with at least 1 fecal culture-positive animal were considered in the herd-level analysis where fecal culturing was used as the reference.

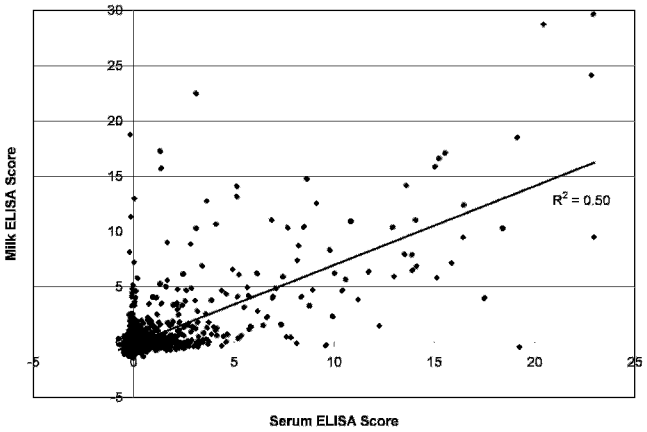

Figure 1. Distribution and correlation of milk and serum ELISA scores (n = 6,349).

Results

Cow level

The average herd size for the 35 operations was approximately 450 head, with a minimum of 25 and a maximum of 1,800 head. Of the 35 operations, 21 had individual animal fecal culture results. Three of the 21 operations had no cattle that tested fecal culture positive, and so were not included in the fecal culture analysis. A total of 6,349 animals had milk and serum ELISA results, and of these, 1,921 animals had fecal culture results. The percentage of matched milk and serum samples that tested positive (including strong positive) was 3.4% (213/6,349) and 4.4% (278/6,349), respectively. The percentage of positive samples for the population of cows where all 3 test results were available, was 3.3% (63/1,921), 3.7% (72/1,921) and 8.8% (170/1,921) for milk, serum, and fecal samples, respectively.

There was significant correlation (R^2 = 0.50) between the milk and serum ELISA scores ($P <$ 0.0001; Fig. 1). Stuart's chi-square test statistic was highly significant ($P < 0.001$), suggesting that serum and milk ELISA-categorized results differed (Table 3). However, overall agreement between the milk and serum ELISAs was moderate on the basis of a weighted kappa value of 0.53.

The relative Se of the milk ELISA, using serum ELISA as the reference, was 45.7 (95% confidence interval [CI] 39.7–51.7) (Table 4). The simple proportion agreement was 0.96, with a test-positive agreement of 0.52 and a test-negative agreement of 0.98. The cow-level Se of the milk and serum ELISAs, relative to fecal culturing was 21.2 (95% CI 15.3–28.1) and 23.5 (95% CI 17.4–30.6), respectively. Simple kappa values for both ELISA comparisons relative to fecal culture results indicated only fair agreement (approx. 0.28). The overall proportional agreement,

Table 3. Comparison of the distribution of cow-level serum and milk MAP ELISA results.

	Serum ELISA			
Milk ELISA	Strong positive	Positive	Negative	Total
Strong positive	55	18	15	88
Positive	18	36	71	125
Negative	15	136	5,985	6,136
Total	88	190	6,071	6,349

Stuart's chi-square test = 18.91, $P < 0.001$. Weighted kappa =0.53 (95% confidence interval = 0.48–0.58).

proportion test positive, and proportion test negative were similar for both ELISAs, compared with fecal culturing. A subset of samples, including those for which milk samples were collected within 60 days of serum and fecal samples was evaluated to determine whether the time between sample collections had an impact on test agreement. Although the relative Se and kappa values estimates were improved when comparing only samples collected within 60 days, the confidence intervals overlapped.

Although the relative Se for both ELISAs was low, compared with that for fecal culturing, the ability of the ELISAs to detect cows shedding MAP in the feces increased as the fecal shedding level increased (Table 5). Greater than 50% of moderate shedders and 70% of heavy shedders were detected by each ELISA method. There were no significant differences in the serum or milk ELISA relative Se at each fecal culture shedding level.

Logistic regression models

Descriptive statistics. Milk and serum ELISA results were available for 8,552 and 6,874 animals, respectively. The percentage of cows testing serum ELISA positive was almost double the percentage of cows testing milk ELISA positive (4.2 and 2.7%, respectively). The largest animal numbers tested were non-Holsteins, had a cow rating > 110%, and were located in the West region (Table 6). Cows that were non-Holsteins had a low cow rating (<80%), were located in the Southeast region and tended to have higher percentages of serum and milk ELISA positive results. A higher percentage of nonlactating cows were serum ELISA positive, whereas cows in the first 2 weeks of lactation more frequently tested positive by the milk ELISA.

Milk ELISA model. The logistic regression model for the milk ELISA response indicated that non-Holstein cows were significantly more likely to test milk ELISA positive, compared with Holstein cows (odds ratio [OR] 2.2, 95% CI 1.6–3.2) (Table 7). Cows in the second and fifth lactations were more likely to test positive, compared with cows in the first lactation (OR 1.4 and 2.6, respectively). Cows in the first 2 weeks of lactation had odds 7.2 times greater for testing milk ELISA positive (95% CI 3.6–14.1) than did cows between 3 and 12 weeks in milk production. Compared with milk production ≤ 80% of herd average, production > 80% of herd average was negatively associated with positive milk ELISA status. There was a significant association between region of the United States and milk ELISA results, with cows in the West and Midwest regions having significantly lower odds of testing milk ELISA positive, compared with cows in the Southeast region.

Two significant interactions were detected in the milk ELISA model: an interaction between breed and lactation number ($P = 0.022$) and lactation number and cow rating ($P < 0.001$. Visual evaluation of interaction graphs revealed no differences in slopes, which coincided with the small coefficients for the interactions. Hosmer-Lemeshow chi-square goodness of fit was not significant when interactions were removed (*P*-value decreased from 0.7 to 0.5). The sign of beta coefficients did not change, and there was no change in significance when the interactions were removed.

Table 4. Cow-level MAP test agreement comparison.

Test	Reference	Group*	Animals/herds	Se	95% CI	Simple kappa	95% CI	Overall proportion agreement (P0)	Proportion positive agreement (P+)	Proportion negative agreement (P−)
ME	SE	All	6,349/35	45.7%	39.7–51.7	0.50	0.44–0.55	0.9627	0.5173	0.9806
		60	2,924/18	53.2%	44.6–61.6	0.59	0.51–0.66	0.9661	0.6024	0.9823
ME	FC	All	1,921/18	21.2%	15.3–28.1	0.27	0.20–0.35	0.9162	0.3090	0.9554
		60	344/8	33.3%	15.6–55.3	0.45	0.24–0.66	0.9477	0.4706	0.9725
SE	FC	All	1,921/18	23.5%	17.4–30.6	0.29	0.22–0.37	0.9157	0.3306	0.9550
		60	344/8	45.8%	25.6–67.2	0.54	0.35–0.73	0.9506	0.5641	0.9738

Se = relative cow-level sensitivity; ME = milk ELISA; SE = serum ELISA; FC = fecal culture.
* Group represents all eligible animals, or those from which milk was collected within 60 days of serum and fecal sample collections.

Table 5. Distribution of milk and serum ELISA results and fecal MAP culture results, by shedding level.

Fecal culture shedding level	Total	Milk ELISA			Serum ELISA			Milk and serum ELISA
		Strong positive (%)	Positive (%)	Negative (%)	Strong positive (%)	Positive (%)	Negative (%)	Positive (including strong positive) (%)
Heavy	13	61.5	15.4	23.1	53.8	38.5	7.7	69.2
Moderate	26	38.5	15.4	46.1	38.5	11.5	50.0	46.1
Low	83	7.2	3.6	89.2	4.8	4.8	90.4	6.0
Very low	48	2.1	4.2	93.7	4.2	10.4	85.4	4.2
Negative	1,751	0.3	1.2	98.5	0.2	1.6	98.2	0.3
Total	1,921	1.6	1.7	96.7	1.4	2.3	196.3	1.8

Serum ELISA model. Lactation number was not significantly associated with the serum ELISA result, although owing to targeted serum testing, only 3.2% of animals tested were in their first lactation, compared with 41.4% of the animals tested by use of the milk ELISA. An association between lactation period (days since calving) and serum ELISA status was not found. A significant association between serum ELISA result and region was revealed; cattle in the West and Midwest regions were less likely to test positive than those in the Southeast region (Table 8). An interaction between milk production and breed was found for animals tested by serum ELISA. Holstein cows that produced ≤80% of herd-average milk production were less likely to test serum ELISA positive than non-Holstein cows that produced ≤80% of herd average. Contrasts indicated that there was no significant difference in the odds of Holstein and non-Holstein cows testing serum ELISA positive for cows that produced >80% of herd-average milk production. The Hosmer-Lemeshow chi-square *P*-value was not significant for the serum model ($P = 0.5$), suggesting good model fit.

Herd level

Apparent within-herd prevalence for both ELISA methods were calculated, and values were between 0 and 13.5% for milk ELISA and 0 and 15.5% for serum ELISA. The apparent prevalence estimates for each of the participating operations are presented in Fig. 2. The Pearson correlation, comparing milk and serum within-herd prevalence, was 0.79 ($P < 0.0001$). The apparent within-herd prevalence for fecal culturing of the 18 infected herds was <1.0 to 27.6%.

Table 6. Percentage of dairy cows testing MAP ELISA positive by logistic regression model variable.

Variable	Level	Total milk samples	Milk ELISA positive (%)	Total serum samples	Serum ELISA positive (%)
All cows		8,552	2.7	6,874	4.2
Breed	Holstein	7,784	2.3	6,186	3.9
	Non-Holstein	768	7.3	688	7.4
Lactation	1	3,537	1.9	220	2.3
	2	2,256	2.6	2,708	3.4
	3	1,411	3.4	1,852	4.2
	4	787	3.8	1,062	5.1
	5	561	5.9	1,032	6.2
Lactation period (weeks since calving)	1–2 wk	233	12.0	208	1.9
	3–12 wk	1,282	2.2	1,065	3.9
	13–28 wk	2,577	1.8	1,616	4.7
	29–44 wk	2,464	2.6	1,534	4.3
	45+ wk	1,996	3.5	1,095	6.2
	Nonlactating	NA	NA	504	6.6
Cow rating*	>110%	3,442	1.8	2,649	2.9
	101–110%	1,862	2.3	1,449	4.7
	91–100%	1,527	2.6	1,165	4.2
	81–90%	924	4.1	811	5.1
	≤80%	797	6.8	800	7.3
Region	West	4,387	1.9	3,905	3.1
	Midwest	1,741	2.1	1,266	3.5
	Northeast	799	2.1	658	3.7
	Southeast	1,625	6.0	1,045	10.0

* Rating as a percentage of herd-average milk production.

Table 7. Results of logistic regression analysis for dairy cows testing milk ELISA positive for MAP antibody.

		Milk ELISA (n = 8,552)				
Variable	Level	Beta coefficient	Standard error	Odds ratio	95% CI	Wald F P-value
Intercept		−3.0329	0.2933	0.05	0.03–0.09	
Breed						0.0001
	Holstein			Referent		
	Non-Holstein	0.8091	0.1804	2.2	1.6–3.2	
Lactation number						0.0319
	1			Referent		
	2	0.3459	0.1598	1.4	1.02–2.0	
	3	0.5079	0.2955	1.7	0.9–3.0	
	4	0.6794	0.3476	2.0	0.97–4.0	
	5 or more	0.9484	0.3186	2.6	1.3–4.9	
Lactation period						<0.0001
	1–2 wk	1.9675	0.3345	7.2	3.6–14.1	
	3–12 wk			Referent		
	13–28 wk	0.0580	0.2830	1.1	0.6–1.9	
	29–44 wk	0.3718	0.2400	1.5	0.9–2.4	
	45+ wk	0.9137	0.3350	2.5	1.3–4.9	
Cow rating*						<0.0001
	>110%	−1.3161	0.1653	0.3	0.2–0.4	
	101–110%	−1.0632	0.1843	0.3	0.2–0.5	
	91–100%	−0.9686	0.1797	0.4	0.3–0.5	
	81–90%	−0.5400	0.1929	0.6	0.4–0.9	
	≤80%			Referent		
Region						0.0073
	West	−0.8491	0.3211	0.4	0.2–0.8	
	Midwest	−0.8589	0.2334	0.4	0.3–0.7	
	Northeast	−0.6145	0.4239	0.5	0.2–1.3	
	Southeast			Referent		

* Rating as a percentage of herd-average milk production.

The relative HSe of the milk ELISA, using serum ELISA as the reference, was 96.3% (26/27 herds) using any test-positive result to classify a herd. Three herds were milk ELISA positive/serum ELISA negative, and 5 herds did not have a single animal test positive by either ELISA. Of the 5 ELISA-negative herds, 3 had individual fecal culture results. The apparent fecal-culture prevalence in the 3 herds was 0.0, 2.6, and 11.8%. McNemars chi-square P-value of 0.32 suggests that the proportions that were positive did not differ.

Twenty-one of the 35 ELISA herds were evaluated by individual fecal culturing. Of these, 18 had at least 1 animal that was fecal-culture positive and were included in the relative HSe calculations. The HSe was 83.3% (15/18 herds) for both ELISAs if any animal tested positive by either ELISA. The relative HSe was the same for milk and serum ELISAs (61.1%) when a 2% apparent prevalence cutoff value was used to classify a herd.

Discussion

We found that the cow- and herd-level relative sensitivities of the milk ELISA were comparable to those of the serum ELISA when fecal culturing was used as the reference. Although it is not a "gold standard," fecal culturing was used as the reference since a currently available antemortem gold standard test is not readily available. The agreement between milk and serum ELISA results was considered moderate on the basis of the kappa statistic.

Kappa values for cow-level analysis suggested that serum and milk ELISA results were only in fair agreement with fecal culture results which, on the basis of published Se and Sp estimates of ELISA methods compared with fecal culture results, was expected. The serum ELISA had relative Se and Sp of approximately 29 and 95–99%, respectively, depending on the manufacturer.[6] When 2% prevalence was used for herd level evaluation, the relative sensitivity of both ELISAs decreased, since some infected herds, on the basis of fecal culture results, had seroprevalence of $< 2\%$.

Two studies[6,13] have indicated similar results with respect to the milk ELISA. However, the relative sensitivity of the milk and serum ELISA, compared with fecal culturing reported in a Canadian study,[13] was higher than the sensitivity reported in this study and a previous study.[6] The manufacturer of the serum ELISA in this study was different from that used in

Table 8. Results of logistic regression analysis for dairy cows testing serum ELISA positive for MAP antibody.

Variable	Level	Serum ELISA (n = 6874)				
		Beta coefficient	Standard error	Odds ratio	95% CI	Wald F *P*-value
Intercept		−1.9525	0.2374	0.1	0.08–0.23	
Breed						NR
	Holstein			Referent		
	Non-Holstein	1.0508	0.2190			
Cow rating*						NR
	>110%	−0.6906	0.1606			
	101–110%	−0.1871	0.1795			
	91–100%	−0.3733	0.1734			
	81–90%	−0.2324	0.1993			
	≤80%			Referent		
Breed by cow rating interaction						<0.0001
	Non-Holstein, >110%	−0.9218	0.3413	0.4	0.2–0.8	
	Non-Holstein, 101–110%	−1.1655	0.3792	0.3	0.1–0.7	
	Non-Holstein, 91–100%	−0.9369	0.2771	0.4	0.2–0.7	
	Non-Holstein, 81–90%	−0.5360	0.3332	0.6	0.3–1.1	
	All other levels			Referent		
Region						0.0007
	West	−1.1465	0.2574	0.3	0.2–0.5	
	Midwest	−1.0898	0.3086	0.3	0.1–0.6	
	Northeast	−0.9746	0.5162	0.4	0.1–1.1	
	Southeast			Referent		

NR = not reported.
* Rating as a percentage of herd-average milk production.

the Canadian study, but the US study[6] evaluated 5 commercially available serum ELISA kits, including the kit used in the Canadian study, and reported a sensitivity of 29%, compared with 74% from the Canadian study. Reported serum and milk ELISA sensitivities for this study were comparable to those of a recently published report.[6] Decreased fecal culturing sensitivity, relative to the methods used in this study, and the use of highly infected herds for the test population could explain why the Canadian study reported such a high ELISA sensitivity.

The results of this study and other recent studies are in contrast to a similar study,[11] from which a kappa of only 0.08 was reported, which represents only slight agreement between tests. The most likely reason for this difference is improvements in milk ELISA methods, and since fecal culturing was not performed, a relative sensitivity could not be calculated for comparison.

The milk ELISA portion of this study was designed and implemented after the NAHMS Dairy 2002 study had begun. The delay in timing of this study didn't allow the collection of milk during the same period as collection of feces and serum from all the participating dairy operations. Even though a substantial period had elapsed for some of the operations, the relative sensitivity and overall test agreement was not different when sample collection was completed within 2 months.

The moderate agreement between the serum and milk ELISA in this study could be attributed to differences in immunoglobulin subclasses and their

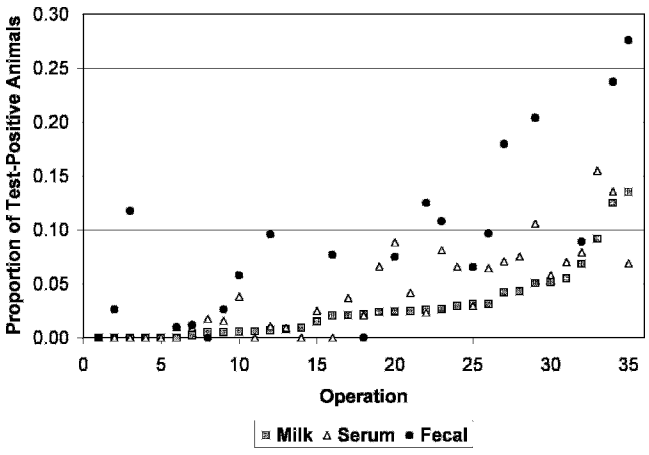

Figure 2. Apparent within–herd-level prevalence for fecal culture shedding and antibodies to *Mycobacterium avium* subspecies *paratuberculosis*, as measured by milk and serum ELISA methods in dairy cows, by operation. Pearson correlation for ELISA apparent prevalence = 0.79 ($P < 0.0001$).

origin. The predominate immunoglobulin in serum, colostrum, and milk from cattle is IgG, which is further classified into three subclasses: IgG1, IgG2, and IgG3.[26] The IgG1 subclass is the predominate IgG in colostrum and milk, but only constitutes approximately 50% of serum IgG. Although a substantial portion of the IgG component of colostrum is derived from active transport of serum IgG, most milk IgG is produced by plasma cells resident in the mammary gland. Additionally, the mammary and intestinal immune systems are not closely linked in ruminants in terms of lymphocyte circulation[12]; since MAP infection is initially localized to the intestine, a response in the milk IgG populations would not be expected. Although the monoclonal antibody used in the milk and serum ELISAs of this study were against IgG1, the different percentages in milk and serum likely affected the ability of the ELISAs to detect anti-MAP antibody.

The odds of testing milk ELISA positive were highest for cows in the first 2 weeks of lactation, compared with that for cows 3 to 12 weeks in lactation (peak milk production), which was in agreement with previous reports.[20,21] Studies have indicated increased milk concentration of IgG and decreased serum IgG concentration at the beginning of lactation, compared with those at other lactation times.[10,31] This is consistent with the production of colostrum, which contains large amounts of antibodies—specifically IgG transported from serum—to provide immunity for the calf. As milk production increases during the course of lactation, fewer antibodies are secreted into milk. The antibodies that are secreted are more likely to be diluted in the increased volume of milk produced.[10] Cows also were more likely to test milk ELISA positive at 45 weeks or more of lactation, as milk production decreases. Results of this study and other studies suggest that milk ELISAs are best performed early or late in lactation, when colostral antibodies are increased or the dilution effect of peak milk production has passed.

The odds of testing milk ELISA positive were significantly lower for cows that produced >80% of herd average milk production than for cows producing <80% of herd average. Although these results support the antibody dilution theory, it has been documented that MAP-infected cows produce less milk than do their noninfected herdmates.[1,2,16] It is difficult to determine whether decreased milk production or increased antibody levels, or both, enable antibodies to be more readily detected in milk in animals with reduced production levels.

Association between testing serum ELISA positive, lactation period, and lactation number was not found. A Danish study[20] not only indicated that the odds of being positive were highest at the end of lactation, but also found an interaction of parity and lactation stage. In the study reported here, we were unable to duplicate these findings, but the small numbers of first-lactation cows may have precluded finding a significant interaction.

The analysis of serum-ELISA status revealed a significant breed and cow rating interaction. Holstein cows may be more resistant to MAP infection than non-Holstein dairy breeds. An English study[3] found an increased risk of MAP infection on operations where Channel Island breeds predominated. The findings of the current study suggest that lower milk producing, non-Holstein cows are more likely to test serum-ELISA positive than lower producing Holstein cows.

The results of this study suggest that, compared with the serum ELISA, the milk ELISA has similar sensitivity and has the potential to be used to identify cattle at high risk for MAP infection without costly visits for collection of serum and fecal samples used in traditional programs. Consequently, herd-level estimates of MAP infection determined by routine, high-throughput analysis of available milk samples could be used to quantify and monitor the risk of individual cattle within DHI-enrolled herds, which represent approximately 45% of the herds and over 50% of all dairy cattle in the USA.[27] Producers currently participating in DHIA testing have already invested in milk sample collection, so additional testing can benefit the producer in lower cost per test. Overall, the milk ELISA performed similarly to the serum ELISA and has the advantage of decreased sample collection costs on farms that use DHIA milk testing.

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Sources and manufacturers

a. Paracheck, Biocor Animal Health, Omaha, NE.
b. BD BACTEC 460TB, Becton, Dickinson and Company, Franklin lakes, NJ.
c. ESP Culture System II, Trek Diagnostic Systems, Cleveland, OH.
d. Sigma Aldrich Chemical Co., St Louis, MO.
e. PANTA/F, Trek Diagnostic Systems, Cleveland, OH.
f. ESP para-JEM broth, Trek Diagnostic Systems, Cleveland, OH.
g. ESP para-JEM GS, Trek Diagnostic Systems, Cleveland, OH.
h. ESP para-JEM AS, Trek Diagnostic Systems, Cleveland, OH.
i. ESP para-JEM EYS, Trek Diagnostic Systems, Cleveland, OH.
j. DNEasy Tissue Kit, Qiagen, Inc., Valencia, CA.
k. Applied Biosystems, Foster, CA.

l. Broad Spectrum Microtabs, D & F Control Systems, Inc., Dublin, CA.
m. Antel BioSystems, Inc., Lansing, MI.
n. Nunc Maxisorp, Fisher Scientific, Pittsburgh, PA.
o. PCDART for Consultants, Dairy Records Management Systems, Raleigh, NC.
p. DHI-Plus for Windows, DHI Computing Service, Provo, UT.
q. Dairy COMP 305 for Windows, Valley Agricultural Software, Tulare, CA.
r. SAS, version 8.2, SAS Institute Inc., Cary, NC.
s. SUDAAN, Release 9.0, 2004, Research Triangle Institute, Research Triangle Park, NC.

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